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Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals

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Abstract Zinc(II) ions are essential for all forms of life. In humans, they have catalytic and structural functions in an estimated 3,000 zinc proteins. In addition, they interact with proteins transiently when they regulate proteins or when proteins regulate cellular zinc re-distribution. As yet, these types of zinc proteins have been explored poorly. Therefore the number of zinc/protein interactions is potentially larger than that given by the above estimate. Confronted with such a wide range of functions, which affect virtually all aspects of cellular physiology, investigators have begun to elucidate the molecular mechanisms of cellular homeostatic control of zinc, especially the functions of transporter, sensor, and trafficking proteins, such as metallothioneins, in providing the correct amounts of zinc ions for the synthesis of zinc metalloproteins. The sulfur-containing amino acid cysteine in proteins has an important role in the cellular mobility of zinc ions. Sulfur-coordination environments provide sufficiently strong interactions with zinc ions; they can undergo fast ligand-exchange; and they can serve as molecular redox switches for zinc binding and release. For the cellular functions of zinc, the free zinc ion concentrations (zinc potentials, $pZn = -\log[Zn^{2+}]$) and the zinc buffering capacity are critically important parameters that need to be defined quantitatively. In the cytoplasm, free zinc ions are kept at picomolar concentrations as a minute fraction of the few hundred micromolar concentrations of total cellular zinc. However, zinc ion concentrations can fluctuate under various conditions. Zinc ions released intracellularly from the zinc/thiolate clusters of metallothioneins or secreted from specialized organelles are potent effectors of proteins and are considered zinc signals. The cellular zinc buffering capacity determines the threshold between physiological and pathophysiological actions of zinc ions. When drugs, toxins, other transition metal ions or reactive compounds compromise zinc buffering, large zinc ion fluctuations can injure cells through effects on redox biology and interactions of zinc ions with proteins that are normally not targeted.

Keywords Zinc · Redox biology · Homeostatic control · Metalloregulation · Metallothionein

Abbreviations

MT	Metallothionein
MTF-1	Metal response element (MRE)-binding transcription factor-1

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Introduction

Among the nutritionally essential transition metal ions, zinc(II) ions have the most pervasive roles in biology, not even matched by those of iron. From prokarya to eukarya, between 4 and 10% of the genomes encode zinc proteins (Andreini et al. 2006a). In human cells, this percentage corresponds to the remarkable number of about 3,000 zinc proteins (Andreini et al. 2006b). This estimate of the size of the human zinc proteome is based on mining sequence databases for zinc-binding motifs that have been established from zinc proteins with known three-dimensional structures (Vallee and Auld 1990). The estimate is conservative, because new coordination environments continue to be discovered and because it is impossible to predict certain types of zinc sites, such as sites in which the binding of the ligands is not sequential (Maret 2004a, 2005, 2008a). Also, zinc ions bind transiently to proteins that are not recognized as zinc proteins. Such interactions include sites where zinc binds between protein subunits or inhibits protein functions, and sites that participate in cellular zinc re-distribution (Maret et al. 1999; Auld 2001; Maret 2004b). Historically, research efforts focused mainly on the roles of catalytic and structural zinc ions in enzymes and other proteins. More recently, the sheer number of zinc proteins has drawn attention to the issue of homeostatic control of this cellular ion. At least several dozen human genes code for cellular proteins involved in this control. They include membrane transporters (exporters and importers from the ZnT (SLC30) and Zip (SLC39) families) that guard trafficking through the cytoplasmic and intracellular membranes (Eide 2006), zinc sensors, such as metal response element (MRE)-binding transcription factor-1 (MTF-1) (Laity and Andrews 2007), and a family of at least 10 functional human metallothionein (MT) proteins (Li and Maret 2008). Major metabolic and signaling pathways control these proteins, indicating that cells integrate zinc metabolism with many of their activities. A major issue regarding the question of how zinc is re-distributed in cells is whether zinc is chaperoned and transferred between proteins by zinc-mediated protein/protein interactions, i.e. without ever being free,[1] or whether zinc ions are generated intermittently from zinc proteins (Heinz et al. 2005). It has been argued that re-distribution of zinc cannot be based on specific chaperone proteins because too many would be required to supply all of the many zinc proteins (Bozym et al. 2006). Yet, the cellular re-distribution of zinc must occur with tight control of the balance between supplying zinc for essential functions and avoiding nonspecific interactions that can cause protein misfolding and toxic effects (Maret 2008b). Characterization of the molecular mechanisms that control the availability of free zinc ions is critical for an understanding of how zinc is transported and how the cell senses whether or not the availability of zinc is adequate.

The discovery of membrane transporters for zinc provided evidence for cellular homeostatic control of zinc and a major impetus for further investigations of the physicochemical basis of how human cells handle zinc ions and how they discriminate between competing metal ions. Both chemical and biological mechanisms are required for proper control. Apparently, every metal ion in its free state is kept at concentrations commensurate with its binding properties (Williams and da Silva 2000). But, in addition to thermodynamic control, metal ion re-distribution is controlled kinetically. The affinity of zinc for cytosolic zinc proteins is picomolar, suggesting that the availability of free zinc ions is quite low. In eukaryotic cells, there are considerable differences in subcellular zinc ion concentrations, because free zinc ions may reach millimolar concentrations in so-called zincosomes and compartments, such as lysosomes. However, total cellular zinc concentrations are a few hundred micromolar, and thus rather high. Free zinc ion concentrations are conveniently expressed as zinc potentials, $pZn = -\log[Zn^{2+}]$, in analogy to pH, the "hydrogen potential." A remarkable development in the zinc biology of eukarya is the observation that free zinc ions can be biological signals (Frederickson 2003). How such zinc signaling works critically depends on how cellular zinc is buffered.

The proteins of cellular zinc homeostasis

By which molecular mechanisms do proteins control cellular zinc homeostasis? Basically, the transport

[1] Free zinc ions have been referred to as "freely available", "labile", or "rapidly exchangeable" zinc that is readily bound to chelating agents. The chemical nature of the ligands of cellular ionic zinc is unknown.

mechanisms of zinc ions through membranes are unknown, though the crystal structure of a bacterial membrane transporter, the Yiip protein of *E. coli*, provides a first glimpse at the architecture and zinc-binding properties of such proteins (Lu and Fu 2007). Yiip is a Zn^{2+}/H^{+} antiporter and belongs to the family of the cation diffusion facilitator (CDF) proteins, which also includes the ZnT proteins. Four zinc ions bind at the interface of the cytoplasmic domain of the homodimer. Additional zinc binding sites are in each transmembrane domain and in a loop connecting two of the five transmembrane helices. The sensing mechanisms for zinc ions are also largely unknown, especially how eukaryotic cells sense low zinc ion concentrations. High zinc ion concentrations are sensed by at least two of the six zinc fingers of MTF-1. The affinities of the fingers for zinc vary about 10–50 fold (Potter et al. 2005). They are in the nanomolar range, and thus lower than for "classic" zinc fingers that typically have affinities in the picomolar range (Laity and Andrews 2007). However, for MT, which is regulated extensively by biological mechanisms, unique chemical mechanisms of how proteins handle zinc ions are emerging. The thiol-based chemistry of MT will be a focus in this article.

Metallothionein and zinc re-distribution

How human cells re-distribute zinc and how they control free zinc ion concentrations is not a trivial issue, because zinc must be mobilized from its tight binding sites in a controlled way so that there is specificity in its actions and minimal interference with the actions of other metal ions. MT participates in this process by serving as a reservoir of cellular zinc (about 10% of zinc in a hepatocyte is bound to MT). Mammalian MT is mostly a zinc protein, and its participation in zinc metabolism has been discussed widely (Kägi 1993; Vallee and Maret 1993; Cousins et al. 2006). Critical to its protein structure and function are twenty cysteine residues (Maret 2008c). The sulfur donor atoms of these cysteines bind up to seven zinc ions in two zinc/thiolate clusters. One cluster has three metals and nine cysteines, Zn_3S_9, a signature that is also found in other proteins (Zhang et al. 2002), and the other has four metals and 11 cysteines, Zn_4S_{11}, a structure that so far has been found only in MT. In humans, at least 10 MT proteins with considerable variation in their primary structures are expressed tissue-specifically (Li and Maret 2008). Formally, all the zinc ions are in a tetrathiol(ate) environment,[2] but since there are only 20 and not 28 cysteines that would be required to bind the metal ions individually, thiolate ligands are shared between zinc ions and form ligand bridges. It was thought that all seven zinc ions are bound with indistinguishable, strong affinity to MT, a paradigm that dominated the field since the discovery of MT 50 years ago. However, this is not the case. In human MT-2, affinities vary over several orders of magnitude, and there are at least three classes of binding sites. One zinc ion is bound rather weakly ($\log K = 7.7$), two are bound with intermediate affinity ($\log K \sim 10$), and four are bound with high affinity ($\log K = 11.8$) (Krężel and Maret 2007a). This heterogeneity is remarkable, since each zinc ion is in a tetrahedral coordination environment with sulfur donors only. Thus the cluster structures de-stabilize rather than stabilize some zinc sites. Another aspect of the sulfur donors is that they confer redox activity on the zinc/thiolate clusters. Generally, the zinc ions in zinc proteins are not redox-active. However, unlike in complexes of other transition metal ions where the central atom is redox-active, in zinc complexes the thiolate ligands confer redox-activity on the coordination environment (Maret and Vallee 1998; Fig. 1). This property allows coupling between proteins with zinc/thiolate coordination environments and redox metabolism in such a way that reducing conditions lower the availability of zinc ions while oxidizing conditions increase it (Maret 2000, 2003, 2006; Fig. 2). In MT, the occupancy of the sites with zinc and the redox state of the sulfur donors are linked: the more zinc is bound the less reactive MT becomes toward thiol-oxidizing or modifying agents. These properties demonstrate that the physiologically important structure of MT is not the one established by crystal and solution structures where seven zinc ions are bound to 20 reduced thiolates (Arseniev et al. 1988; Robbins et al. 1991). Instead, the structure of MT depends on the availability of zinc ions and the

[2] Two protons are released when four cysteinyl side chains react with a zinc(II) ion. Whether or not the remaining two thiols ionize to thiolates depends on the pH value and hydrogen bonding, among other factors.

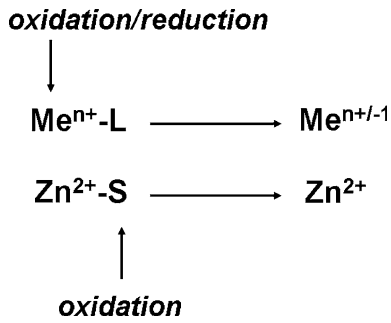

Fig. 1 Strategies for metal ion mobilization. In many redox-active transition metal ions, the central atom undergoes oxidation/reduction thereby changing its binding properties. In contrast, oxidation of the sulfur donor atom of the cysteine ligand causes zinc dissociation in biological zinc/thiolate coordination environments

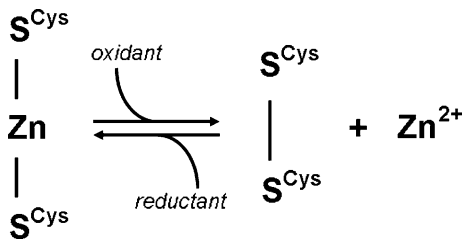

Fig. 2 A redox zinc switch. Cysteine/cystine interconversion can control zinc release and binding, a molecular mechanism that links zinc and redox metabolism

redox poise in the cellular environment. Differential chemical modification with thiol-reactive probes in tissues and cells has shown that fractions of MT are in the form of the apoprotein (thionein) and the oxidized protein (thionin) (Yang et al. 2001; Krężel and Maret 2007b). Thionein contains disulfides and exists in polymerized forms (Feng et al. 2006; Haase and Maret 2008). In contrast to other zinc proteins where the zinc binding sites are fully metalated, the sites in MT are not fully occupied (Krężel and Maret 2008). Based on its variable redox state and zinc binding, the properties of MT are consistent with that of a redox protein and with a regulatory role in zinc metabolism rather than a thermodynamic sink for zinc (Fig. 3).

Although cytoplasmic zinc chaperones are unknown, it is clear that cellular zinc concentrations are controlled tightly (Nies 2007). Understanding the molecular mechanisms of cellular and subcellular zinc transport and the binding processes that govern zinc/protein interactions is a prerequisite for understanding

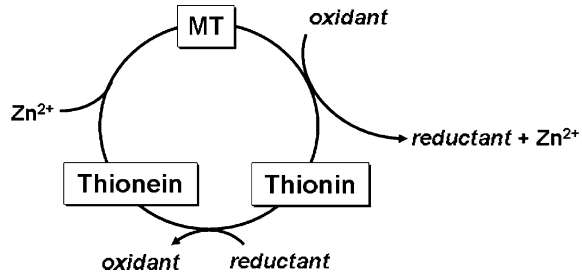

Fig. 3 Metallothionein redox cycle. In this cycle, one redox couple controls zinc release from MT, the zinc-loaded state, and generates a partially oxidized and zinc-loaded state (thionin). The same or another redox couple restores the reduced protein (thionein) for subsequent loading with zinc ions

the large number of cellular functions that depend on zinc. For tight regulation, proteins need to chaperone zinc, and multiple forms of MT are leading candidates for such a function. The term metallochaperone has been introduced for proteins involved in copper metabolism (O'Halloran and Culotta 2000). It describes the characteristics of proteins that interact specifically with their targets, the apoproteins, and transfer metal ions by an associative mechanism. Although evidence for such a mechanism has been presented for zinc exchange between different MTs and between MT and other zinc finger proteins (Otvos et al. 1993; Maret et al. 1997), it apparently does not involve recognition through complementary protein surfaces, a hallmark of copper chaperones. Therefore, it is a matter of how strictly one defines the term metallochaperone if one were to call MT a zinc chaperone. MT certainly is a metal-trafficking protein. It is transported from the cytoplasm to the intermembrane space of mitochondria or to the nucleus (Tsujikawa et al. 1991; Ye et al. 2001), two cellular compartments, for which there are no known zinc transporters. MT is also taken up by cells through a receptor-mediated endocytotic pathway, suggesting additional functions in cellular zinc acquisition and/or communication (Erfurt et al. 2003; Hao et al. 2007).

Free zinc ion concentrations (zinc potentials) and the cellular zinc buffering capacity

Estimates of free zinc ion concentrations in eukaryotic cells were made by various methods since as early as 1971 and were in the range of hundreds of picomolar in rabbit skeletal muscle (Peck and Ray

1971); 24 pM in erythrocytes (Simons 1991); and 500 pM in neuroblastoma cells (Adebodun and Post 1995; Benters et al. 1997). More recent estimates range from femtomolar in bacteria (Outten and O'Halloran 2001) to micromolar in eukaryotic cells (Brand and Kleineke 1996). Highly sensitive fluorescent zinc ion sensors that can be introduced into cells made it possible to measure free zinc ions. The lowest estimates are 5–10 pM for rat pheochromocytoma (PC12) cells (Bozym et al. 2006). A concentration of 1.07 nM was measured in rat primary cortical neurons (Colvin et al. 2008). Introducing a zinc ion sensor into a cell affects the cellular zinc buffering capacity and can generate a bias in the measurements, resulting in lower apparent free zinc ion concentrations (Krężel and Maret 2006). Measurements at different concentrations of the sensor and extrapolation to a zero concentration of the sensor eliminate any contribution of the sensor to cellular zinc buffering. Employing this procedure, a free zinc ion concentrations of 784 pM was measured in the human colon cancer HT-29 cell line (Krężel and Maret 2006), a value that is remarkably similar to the early estimates cited above.

The cellular zinc buffering capacity was also determined in HT-29 cells (Krężel and Maret 2006). It comprises 28 μM of ligands that are not saturated with zinc and have high affinity for zinc.[3] This concentration corresponds to about 10% of the total ligands, because the total zinc concentration is 264 μM in these cells. About one-third of the 28 μM of ligands are thiols (Krężel et al. 2007). If this combined zinc and redox buffering capacity of the thiols is lowered by oxidation, chemical modification or association with toxic metal ions, free zinc ion concentrations increase and induce thionein, the apoprotein of MT, and thus increase the zinc buffering capacity. Only under conditions of its induction does MT contribute significantly to the zinc and redox buffering capacity (Krężel and Maret 2007b). Free zinc ions can also be sequestered in cellular organelles, a mechanism that complements physicochemical buffering and has been referred to as muffling (Thomas et al. 1991). If the capacity to induce MT is exhausted, higher and potentially cytotoxic zinc ion concentrations occur. The properties of the cellular zinc buffering capacity suggest new pathways of how cellular zinc is controlled, how zinc ions participate in cellular control, and how they can injure cells in diseases that are accompanied by oxidative or environmental stress, particular genetic dispositions, or nutritional zinc deficiencies (Maret and Krężel 2007). Because cellular zinc buffering capacity is redox-sensitive and rather limited, some cells are rendered vulnerable to damage by larger zinc ion signals when buffering is compromised under conditions, such as oxidative stress. Environmental agents can also affect zinc buffering and elicit zinc ion fluctuations beyond their usual amplitudes. In this way, zinc ions become potent effectors of the native and non-native structures of proteins. Zinc buffering can be perturbed by redox agents that modify zinc-buffering ligands, xenobiotics that bind zinc or cysteinyl sulfur, or thiophilic metal ions, such as cadmium, mercury or lead, which either bind to potential zinc ligands or displace zinc from its binding sites (Fig. 4). Other classes of agents that increase free zinc ion concentrations are reducible selenium compounds and endogenous reactive carbonyls formed during oxidative stress, lipid peroxidation, hyperglycemia-induced glycations and environmental exposures (Jacob et al. 1999; Chen and Maret 2001; Hao and Maret 2006). To summarize, the zinc buffering capacity determines free zinc

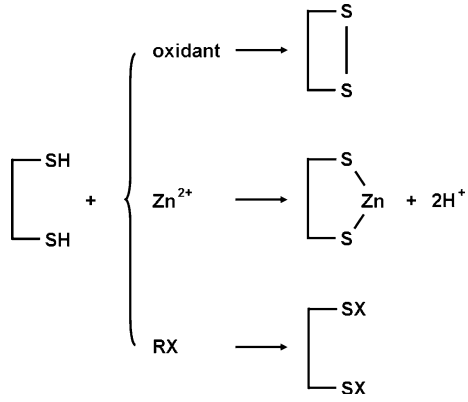

Fig. 4 Thiols as a fraction of the cellular zinc buffering capacity. Oxidants and other thiol-reactive compounds, such as reactive species and carbonyls, compete with thiols for zinc binding, thereby lowering the buffering capacity and increasing the availability of zinc ions

[3] While the overall zinc buffering capacity of a cell is high, this particular zinc buffering capacity is limited and maintains physiological pZn. This feature may be illustrated by comparison with polybasic acids where different ionizations provide buffering in different pH ranges.

ion concentrations, the fluctuations of which are the basis for zinc signaling.

Physiological and pathophysiological fluctuations of free zinc ions (zinc signals)

Zinc ions have been discussed first as second messengers over 20 years ago (Grummt et al. 1986). There is renewed interest in this topic, because free zinc ions are released from presynaptic nerve terminals and affect the physiology of the postsynaptic neuron (Frederickson et al. 2005). They are also released intracellularly either from zinc/thiolate coordination environments or from intracellular stores (Maret 2004c; Yamasaki et al. 2007). The role of zinc ions in biological regulation and pathological mechanisms is becoming a significant theme in cell signaling. Zinc ion fluctuations can be induced by stimulation of excitable cells, DNA-damaging agents, cellular influx of zinc, mitochondrial zinc release (Atar et al. 1995; Choi and Koh 1998; Smith et al. 2002; Sensi et al. 2003), and by release from zinc proteins when cells are exposed to oxidizing agents or when oxidative signals are generated within cells (Turan et al. 1997; Aizenman et al. 2000; St. Croix et al. 2002; Spahl et al. 2003; Cima et al. 2006). A few selected examples will serve to demonstrate how zinc ions released intracellularly from proteins with zinc/thiolate coordination environments, such as MTs, become messengers in a fundamental molecular pathway, in which a redox signal is converted into a zinc signal:

$$\text{Redox signal} \rightarrow \text{proteins with zinc/thiolate coordination} \rightarrow Zn^{2+} \rightarrow \text{target(s)}.$$

Changes in MT have been implicated in the control of cellular zinc ion fluctuations. For example, MT can be thionylated (Maret 1994). Upon homocysteinylation, MT no longer scavenges superoxide ions and the released zinc ions activate the zinc finger transcription factor Egr-1 (Barbato et al. 2007). Such an impairment of zinc and redox homeostasis is believed to be a mechanism of how hyperhomocysteinemia, a major risk factor for heart disease and stroke, increases production of reactive species and may cause chronic inflammation, and atherothrombotic disease (Colgan and Austin 2007). MT also can be nitrosylated (Kröncke et al. 1994). Agents that increase intracellular calcium in endothelial cells release zinc from MT through a nitric monoxide (NO)-mediated mechanism (Pearce et al. 2000). In this case, the released zinc ions activate MTF-1, which controls the expression of genes involved in the antioxidant defense (Stitt et al. 2005). While this NO-MT-Zn^{2+} pathway is cytoprotective in the lung (Bernal et al. 2008), it can be cytotoxic in the brain. Thus, calcium influx into neurons activates synthesis of NO and superoxide, both of which combine to form peroxynitrite and release cytotoxic levels of zinc ions (Bossy-Wetzel et al. 2004). These experiments reinforce the need to quantify zinc ion fluctuations and zinc buffering that determines the borderline between physiology and pathophysiology (Maret 2008b).

Not only does the redox state affect zinc ion concentrations via zinc/thiolate coordination environments, but free zinc ion concentrations are directly related to the cellular redox state. Zinc is considered an antioxidant because it protects the cell against oxidative damages (Bray and Bettger 1990; Powell 2000). However, antioxidant functions of zinc must be indirect, i.e. pro-antioxidant, because zinc ions do not participate in redox reactions in biology (Hao and Maret 2005). Moreover, the outcome depends on the concentrations of free zinc ions: High concentrations elicit an oxidative stress and thus constitute a pro-oxidant condition. Zinc deficiency also induces an oxidative stress (Oteiza et al. 1995; Hennig et al. 1999). Thus, free zinc ion concentrations are a critical parameter for the cellular redox state, and it appears that the redox-inert zinc ion has been chosen to regulate specific aspects of redox metabolism.

Because zinc ion fluctuations occur at such low concentrations and zinc interacts strongly with proteins, released zinc ions are potent intracellular signals. Picomolar to low nanomolar concentrations of zinc ions inhibit enzymes in energy metabolism, signaling, and mitochondrial respiration (Maret et al. 1999; Hogstrand et al. 1999; Ye et al. 2001; Gazaryan et al. 2002; Haase and Maret 2003). Of similar significance for possible regulatory functions of zinc is the increasing number of zinc sites that bridge protein subunits, suggesting that changes in the availability of zinc ions affect protein/protein interactions and perhaps even control them (Maret 2004b). These findings expand the repertoire of zinc

functions beyond that in thousands of zinc proteins, because they identify interactions of zinc with proteins that were not considered to be zinc metalloproteins.

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